

FEDEROFF DECLARATION

Exhibit O

Regulated high level expression of a human γ -globin gene introduced into erythroid cells by an adeno-associated virus vector

(parvovirus/hemoglobinopathy/transfection/hypersensitive site)

CHRISTOPHER E. WALSH[†], JOHNSON M. LIU[†], X. XIAO[‡], NEAL S. YOUNG[†], ARTHUR W. NIENHUIS^{†§},
AND RICHARD JUDE SAMULSKI[‡]

[†]Clinical Hematology Branch, Building 10, Room 7C103, National Heart Lung and Blood Institute, 9000 Rockville Pike, Bethesda, MD 20892; and
[‡]Department of Biological Sciences, Room 269, Crawford Hall, University of Pittsburgh, Pittsburgh, PA 15260

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ABSTRACT Gene therapy of severe hemoglobinopathies will require high-level expression of a transferred globin gene in erythroid cells. Distant regulatory elements flanking the β -globin gene cluster, the locus control region, are needed for appropriate expression. We have explored the use of a human parvovirus, the adeno-associated virus (AAV), for globin gene transfer. The human γ -globin gene, linked to hypersensitivity site 2 from the locus control region of the β -globin gene cluster, was subcloned into a plasmid (psub201) containing the AAV inverted terminal repeats. This construct was cotransfected with a helper plasmid containing trans-acting AAV genes into human 293 cells that had been infected with adenovirus. The recombinant AAV vector containing hypersensitivity site 2 stably introduced on average one or two unrearranged proviral copies into human K562 erythroleukemia cells. The transferred globin gene exhibited normal regulation upon hemin induction of erythroid maturation and was expressed at a level equivalent to a native chromosomal γ -globin gene.

The molecular defects in sickle cell disease and β -thalassemia have been well characterized and seem amenable to genetic correction (1-3). Effective genetic therapy of these hemoglobinopathies requires safe, efficient, and stable transfer of globin genes into human hematopoietic stem cells and subsequent high-level gene expression in maturing erythroid cells. In homozygous patients with β -thalassemia, deficient or absent β -globin gene synthesis causes the production of poorly hemoglobinized, defective erythrocytes resulting in severe anemia (1, 2). Transfer and high-level expression of a normal β -globin gene should be highly effective in correcting the defect. In sickle cell anemia, the mutant hemoglobin is susceptible to polymerization resulting in altered erythrocyte rheological and membrane properties leading to vasoocclusion. Increased fetal hemoglobin production appears to ameliorate the severity of sickle cell disease (3, 4). Production of γ -globin, the unique component of fetal hemoglobin, in erythroid cells of patients with sickle cell anemia may therefore be therapeutic.

Strategies for generation and use of recombinant retroviral vectors are most advanced among the various available viral vector systems for human gene therapy (5). Retroviral vectors have been used to transfer the β -globin gene into murine hematopoietic stem cells (6-8). The human β -globin gene is expressed in most animals but only at a level 1-2% that of mouse chromosomal β -globin genes, a level too low to be of any therapeutic value.

The recently discovered regulatory elements that flank the globin gene cluster offer an additional avenue for the design of viral vectors (9). These regulatory elements are defined by

DNase I hypersensitive sites (HS) and are collectively termed the locus control region (LCR) (reviewed in ref. 10). Four sites (5' HS1 to -4) are located several kilobases (kb) 5' to the ϵ -globin gene and one site (3' HS1) is mapped 3' to the β -globin gene. The active elements of the LCR are encompassed within 300-400 base pairs (bp) of DNA found at each HS (11-15). The 5' HS2, -3, and -4 when linked to globin genes singly or in combination substantially increase globin gene expression in transfected erythroleukemia cells or transgenic animals (10, 16, 17). Extensive efforts to develop retroviral vectors containing globin genes with the regulatory elements needed to achieve high-level expression have been largely unsuccessful (ref. 18; K. McDonagh, B. Sorrentino, and A.W.N., unpublished observations). Indeed, retroviral vectors have as yet been shown to have only limited ability to transfer genes into primate pluripotent hematopoietic stem cells (19), the target for genetic therapy of hemoglobin disorders. These considerations prompted us to explore the recombinant adeno-associated virus (rAAV) vector system for globin gene transfer.

AAV-2 is a defective parvovirus (reviewed in refs. 20 and 21), requiring coinfection with either adenovirus (22, 23) or herpes simplex virus (24) for efficient replication and lytic infection. When no helper virus is available, AAV infection results in the persistence of the AAV genome in the host cell genomic DNA as an integrated provirus (25, 26). Recent characterization of wild-type AAV integration has uncovered specific targeting into the long arm of human chromosome 19 (27, 28). Such targeted integration is unique among the eukaryotic DNA viruses. Subsequent superinfection of latently infected cells by helper virus results in rescue and replication of AAV, thus completing the life cycle (20, 21, 29). The wild-type AAV-2 genome is 4680 nucleotides (nt) long and contains three promoters that control the genes required for replication and encapsidation of the AAV genome (20, 21). Flanking the AAV coding sequences are two 145-nt inverted terminal repeat (ITR) sequences which are the minimal cis-acting elements necessary for replication, encapsidation, integration, and rescue from the host cell genome (X.X. and R.J.S., unpublished data). These ITRs appear to contain no dominant transcriptional regulatory sequences that might interfere with internally placed promoter or enhancer elements as has been observed with retrovirus long terminal repeats. Additional major advantages of AAV transduction vectors are nonpathogenicity of the parent virus and the broad range of host cells that may become latently infected (human, monkey, canine, and murine) (20, 21, 29, 30).

Abbreviations: AAV, adeno-associated virus; rAAV, recombinant AAV; ITR, inverted terminal repeat; LCR, locus control region; HS, hypersensitivity site; nt, nucleotide(s).

[§]To whom reprint request should be addressed.

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AAV vectors have been developed that provide a viable alternative to currently utilized vector systems (31–36). In our system, AAVs are generated from a plasmid (psub201) composed of the cis-acting AAV terminal repeats (ITRs) flanking the gene and regulatory sequences to be inserted into target cells (36). A helper plasmid (pAAV/Ad) supplies complementing gene products for AAV replication and capsid production. This system allows production of rAAV stocks, which are free of contaminating wild-type virus. We have explored the use of rAAV for transfer and expression of the human α -globin gene, linked to a regulatory component of the LCR.

MATERIALS AND METHODS

Viruses and Cells. Human adenovirus type 5 was obtained from the American Type Culture Collection. K562 cells, a laboratory stock previously found to be triploid at chromosome 11 (37), Detroit 6 cells, and 293 cells were maintained in culture as described (36).

Plasmids and DNA. A plasmid containing the ITRs of the AAV genome (psub201) and the complementing plasmid pAAV/Ad containing the entire coding sequence of AAV flanked with adenoviral terminal repeat sequences have been described (36). The plasmid pUC008 contains a polylinker consisting of 5' *Nhe* I, *Xba* I, *Sal* I, *Bgl* II, *Xho* I, *Apa* I, *Bam*HI, *Xba* I, *Nhe* I 3'. The polylinker was subcloned into pUC9 between *Hind*III and *Eco*RI sites. A neomycin-resistance transcription unit (*Neo*^R) driven by a herpes thymidine kinase promoter, isolated from plasmid pMC polA (Stratagene) as an *Xho* I/*Hind*III fragment, was subcloned into pUC008 (pUC008/*Neo*).

Construction of rAAV Plasmids. A *Hind*III/*Xba* I (11) fragment of the LCR HS2 site and the marked α -globin gene (37) were concurrently ligated and subcloned into pUC008/*Neo*. The construct was digested with *Nhe* I and ligated to the psub201 vector that had been digested with *Xba* I. The final vector was termed pAAV/HS2/ α -globin/*Neo* (Fig. 1). All fragments were subcloned in the same 5' to 3' orientation in psub201. The plasmid pAAV/ α -globin/*Neo* was constructed similarly but without the HS2 fragment.

Generation and Characterization of rAAV. Dishes (10 cm²) containing 80% confluent 293 cells were infected with adenovirus type 5 at a multiplicity of 5–10 plaque-forming units per cell as described (36). rAAV viral stocks were generated by subsequent calcium phosphate cotransfection of 10 μ g of plasmid pAAV/HS2/ α -globin/*Neo* or pAAV/ α -globin/*Neo* and 10 μ g of helper plasmid (pAAV/Ad) 2–4 hr after adenoviral infection. Cells were harvested 40 hr posttransfection, frozen and thawed four times, heat treated (56°C, 1 hr) to inactivate adenovirus, and centrifuged to remove denatured debris.

We examined our recombinant stocks for evidence of wild-type AAV virus; the wild-type genome was not detected in DNA extracted from cell lysates (38) when analyzed by Southern blotting (data not shown). Aliquots of lysates (0.01–0.50 ml) were added to 2×10^5 Detroit 6 or HeLa cells in 10 ml of medium and were allowed to incubate overnight. Cells were split and 24 hr later Geneticin (G418; 0.5 mg/ml active; GIBCO) was added. Drug-resistant colonies were isolated 10–14 days later. The rAAV titer was calculated from the number of resistant colonies times the dilution of the stock and averaged 10^4 – 10^5 *Neo*^R infectious particles per ml. No G418-resistant colonies were observed with noninfected cells.

rAAV Infection of Erythroleukemia Cells. rAAV lysate (0.05–0.10 ml of 1×10^4 *Neo*^R colonies per ml) was added to 5×10^5 K562 cells in 10% fetal calf serum/Dulbecco's modified Eagle's medium with glutamine and antibiotics for 24–48 hr. Cells were harvested, washed, and resuspended at 5×10^3 cells per well (24-well plate) in the presence of active

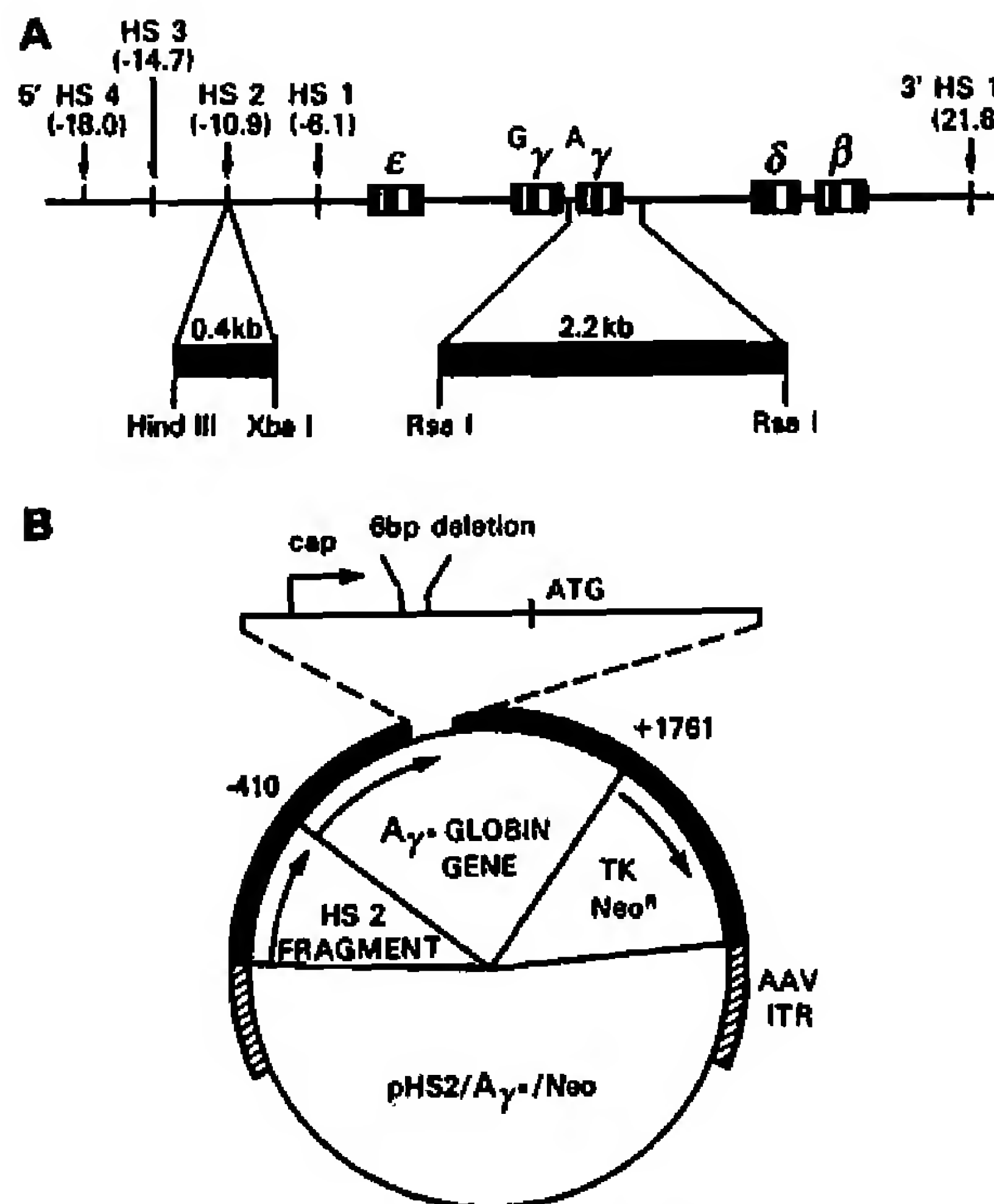


FIG. 1. Construction of the rAAV plasmid pAAV/HS2/ α -globin/*Neo*. (A) Schematic representation of the human β -globin cluster. The five functional genes (ϵ , $G\gamma$, $A\gamma$, δ , and β) are indicated by boxes. Arrows show location of the five major DNase I HS at distances (kb) from the ϵ -globin gene. The HS2 fragment and the α -globin gene used in the vector construction are indicated. (B) HS2 fragment, marked α -globin gene with 6 bp deleted at +23 to +28 relative to the Cap site, and *Neo*^R gene were subcloned into psub201 (36).

G418 (0.5 mg/ml). Control (noninfected) K562 cells yielded no viable cells after drug selection. Drug-resistant colonies were harvested 10–14 days after infection. Thirty individual colonies were isolated, pooled, and grown in a Costar flask. One-half of each pool or clone was grown in 20 μ M hemin (Sigma); both induced and uninduced cells were cultured for 3 additional days.

Analysis of Globin Gene Expression. RNA was extracted from transduced *Neo*^R K562 cells as described (37). An RNase protection assay was performed with a probe transcribed from a subcloned fragment of the native α -globin gene (37). The transcript from the native globin gene protected a 145-nt fragment, whereas the transcript from the mutationally marked globin gene protected a 117-nt fragment. A previously described reverse PCR method (37) distinguished wild-type and marked globin gene transcripts as 96- and 90-bp products, respectively, using primers within the first exon. Primer-extension analysis was performed to map the transcriptional start site as described (14).

Southern Blots. Gene copy number was determined by genomic digestion and Southern blotting (39). Using ammonium acetate buffer (40), the digested DNA was transferred to Hybond N+ (Amersham) and probed with either an *Xho* I/*Pvu* II (840 bp) or an *Xho* I/*Eco*RI (1700 bp) ³²P-labeled fragment of the α -globin gene. Filters were washed to a final stringency of $2 \times$ SSC (standard citrate saline) at 65°C for 1 hr. Autoradiography was performed and the bands of interest were quantitated using the Zeineh soft laser densitometer (LKB).

RESULTS

Transduction of Erythroleukemia Cells with rAAV. K562 cells provide a model for study of globin gene regulation and

have been used to define important cis-acting control elements within the β -globin LCR (41, 42). Addition of hemin induces erythroid maturation of K562 cells, resulting in increased expression of γ -globin genes. Recombinant virus carrying both a marked $\Delta\gamma$ -globin and Neo^R gene were generated as described and used to infect K562 cells. Neo^R clones were obtained and characterized by genomic blotting for both copy number and configuration of the transduced globin gene. As shown in Fig. 2, rAAV/HS2/ $\Delta\gamma^*$ /Neo consistently transduced one or two unrearranged copies of the viral genome into both pooled populations and individual clones. Further restriction analysis of DNA from several clones generated data suggesting random integration (data not shown). All DNAs were negative after performing polymerase chain reaction analysis for the specific region of chromosome 19 used for wild-type AAV integration (28) (data not shown). To further characterize these cells, transduced K562 clones were challenged with adenovirus and low molecular weight DNA was extracted (38) and subjected to Southern analysis. No signal hybridizing to either wild-type AAV sequences or recombinant sequences was detected (data not shown). In the absence of AAV trans-acting factors, the rAAV/HS2/ $\Delta\gamma^*$ /Neo genome remained stably integrated even after adenoviral challenge.

When Southern blot analysis of DNA extracted from a pool of rAAV/ $\Delta\gamma^*$ /Neo^R-infected clones was analyzed, some clones appeared to contain a rearranged proviral genome. A number of possible explanations (generation of a variant during virus replication, efficiency of encapsidating subgenomic vs. rearranged full-length templates, etc.) remain to be investigated. Regardless of the reason, after characterizing several individual clones, we identified cells that contained an intact copy of the viral genome (Fig. 2). Various transduced cell lines were then used to measure globin gene expression with and without the HS2 element.

RNA Expression of the Transduced $\Delta\gamma$ -Globin Gene. The influence of the HS2 element on gene expression is shown in Fig. 3. An RNase protection assay was used to detect the transcript from the chromosomal γ -globin gene and that from the transferred globin gene. RNAs from two pools of 30 individual clones of rAAV/HS2/ $\Delta\gamma^*$ /Neo-infected K562 cells gave a strong signal for the transcript derived from the transferred globin gene. Hemin induction increased expression of both the chromosomal and transferred globin genes

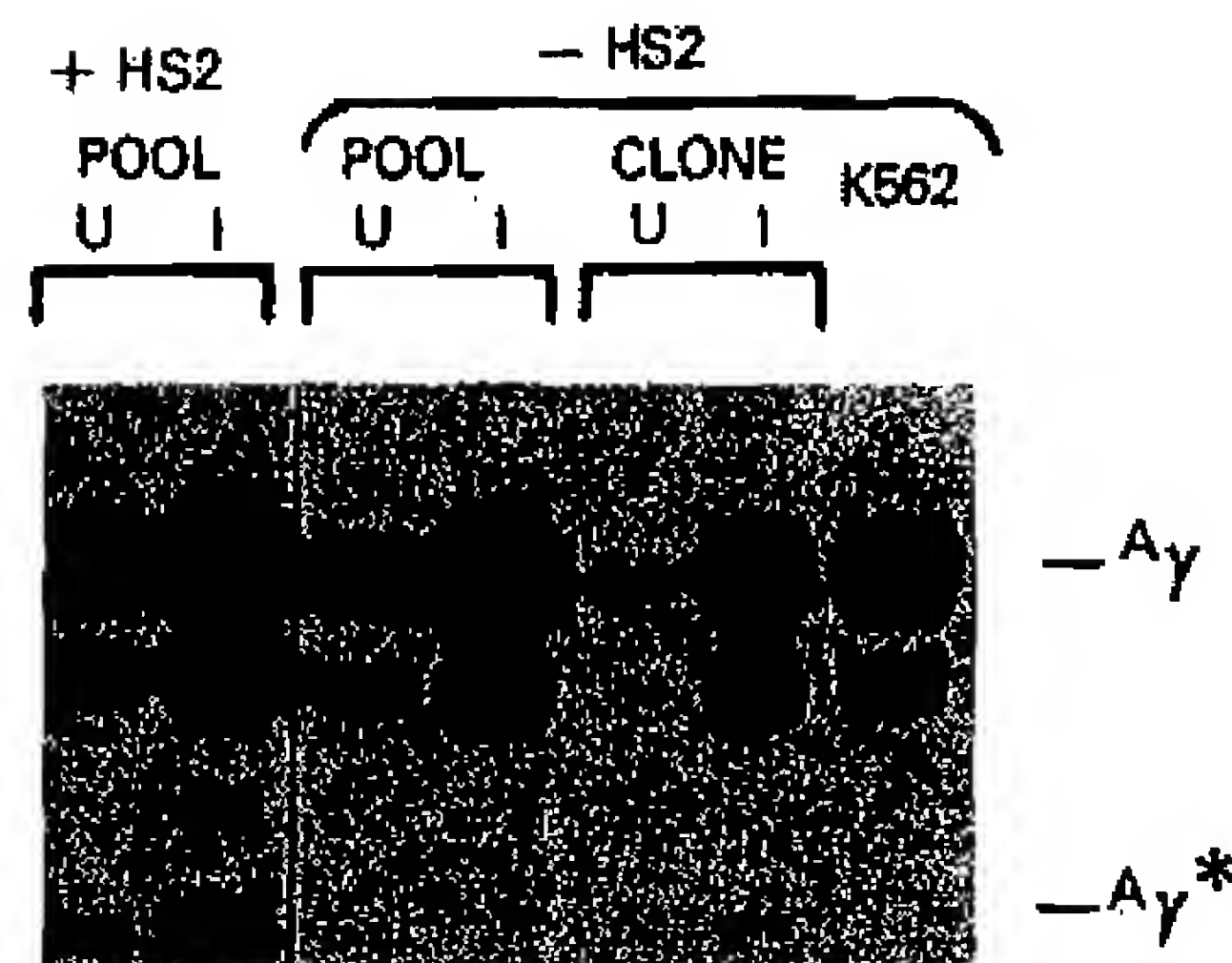


FIG. 3. RNase protection assay of RNA extracted from K562 cells infected with rAAV/HS2/ $\Delta\gamma^*$ /Neo or rAAV/ $\Delta\gamma^*$ /Neo. A pool of rAAV/ $\Delta\gamma^*$ /Neo-infected cells and one clone lacking the HS2 element were analyzed as described (37). RNA from mock-infected cells was run as a control. Predicted protected fragments were 145 nt for the endogenous γ -globin genes ($\Delta\gamma$ and $\Delta\gamma^*$) and 117 nt for the marked transduced gene. The probe used was an 881-nt fragment from the wild-type $\Delta\gamma$ gene (37). U, uninduced cells; I, induced cells.

equivalently. The transcript from the transferred globin gene was initiated at the normal transcriptional start site (Cap) as documented by primer-extension analysis (data not shown). In contrast, the transcript from the transferred globin gene could not be detected in RNA from the pool of cells derived by infection with rAAV/ $\Delta\gamma^*$ /Neo, and the individual clone containing an unrearranged copy of the proviral genome had a very low level of marked γ -globin mRNA (Fig. 3).

The relative amount of the transcript from the chromosomal γ -globin genes and that from the transferred gene was accurately estimated in several individual clones transduced with rAAV/HS2/ $\Delta\gamma^*$ /Neo^R using amplification by the polymerase chain reaction (37). Primers were designed to flank the marked mutation so that the individual transcripts could be distinguished by 8% polyacrylamide gel electrophoresis. Shown in Fig. 4 are data from seven clones with and without hemin induction. In every case, the transferred γ -globin gene exhibited increased expression after hemin induction that paralleled that of the native chromosomal globin genes. Using a correction for the chromosomal gene copy number (six copies), we estimated the relative expression of the trans-

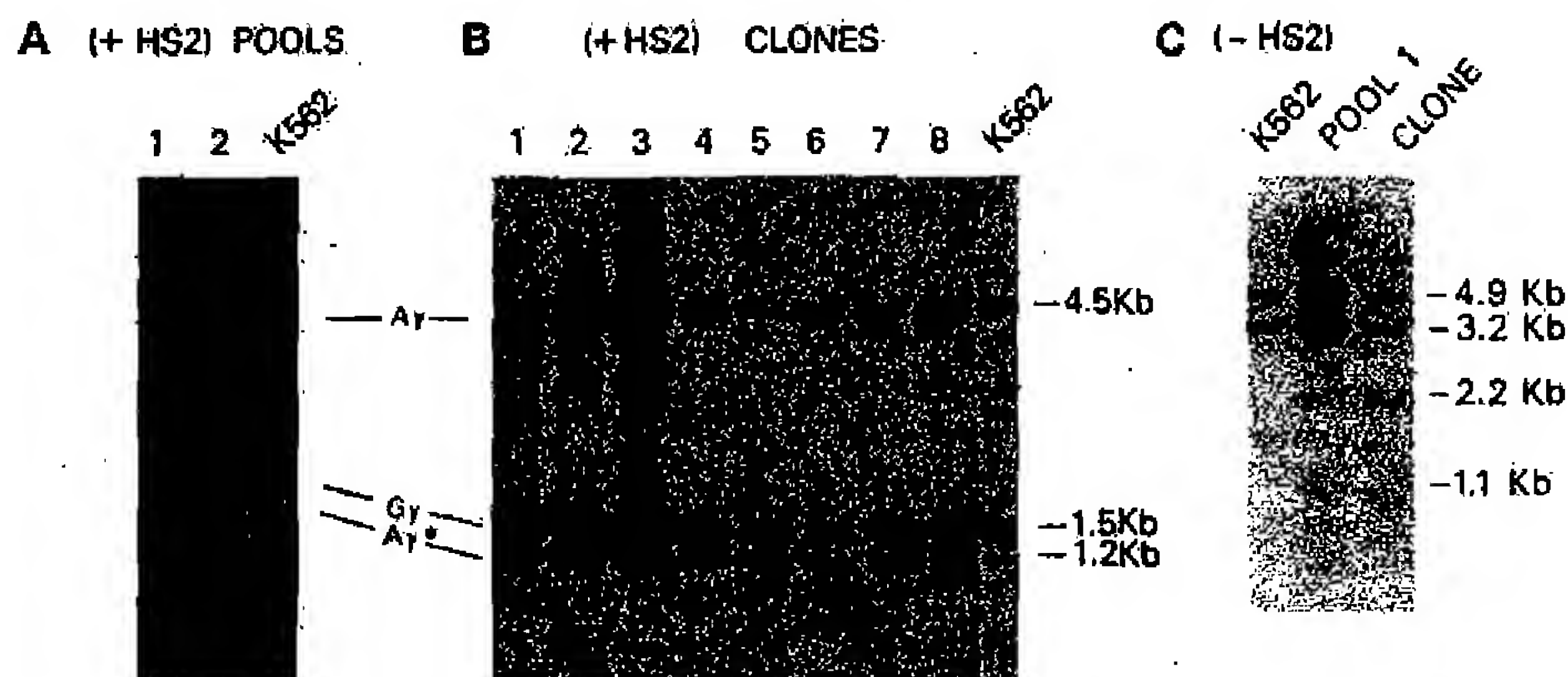


FIG. 2. Southern analysis of DNA from K562 cells infected with rAAV. (A) Southern analysis of two pools of 30 individual clones of cells infected with recombinant AAV virus containing the HS2 fragment (rAAV/HS2/ $\Delta\gamma^*$ /Neo). DNA was digested with *Pvu* II. Bands containing the endogenous $\Delta\gamma$ and $\Delta\gamma^*$ -globin signals migrate at 4.5 and 1.5 kb, respectively. The expected 1.2-kb band represents the HS2-containing construct using an *Xho* I/*Pvu* II $\Delta\gamma$ -globin probe. K562 mock-infected cells served as a source of control DNA. (B) Southern analysis of individual clones infected with rAAV/HS2/ $\Delta\gamma^*$ /Neo. After densitometric analysis, the proviral copy number was calculated by multiplying the signal ratio of transduced $\Delta\gamma^*$ /endogenous γ -globin by 3. (C) Southern analysis of a pool of 30 clones and a single clone generated by infection of K562 cells with the recombinant virus lacking the HS2 fragment (rAAV/ $\Delta\gamma^*$ /Neo). DNA was digested with *Xba* I. Bands containing the endogenous $\Delta\gamma$ and $\Delta\gamma^*$ genes migrate at 4.9 and 3.2 kb, respectively. The expected 1.1- and 2.2-kb bands are derived from the construct lacking HS2 detected with an *Xho* I/*Eco*RI $\Delta\gamma$ probe.

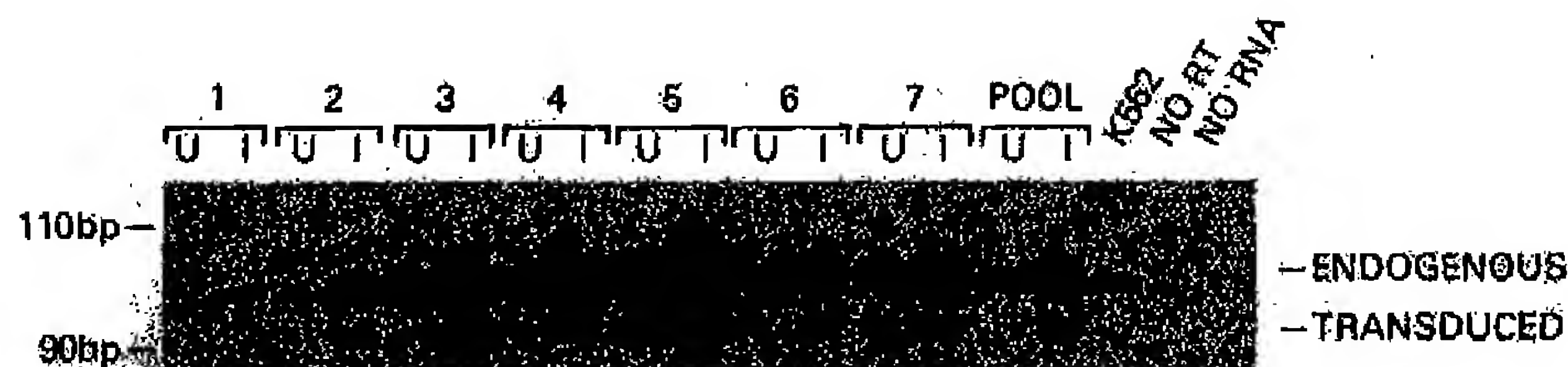


FIG. 4. Expression of globin mRNA in rAAV/HS2/ γ^* /Neo clones measured by the polymerase chain reaction (37). (A) Data from seven isolated clones and a pool of cells are shown. U, uninduced cells; I, induced cells. Controls in the third lane from right are assays of RNA from noninfected K562 cells. RNA processed identically without reverse transcriptase (RT) and samples without RNA served as negative controls. Predicted 90- or 96-nt products are generated by using reverse-transcribed cytoplasmic RNA from either the transduced marked γ^* -globin or the endogenous γ -globin and δ -globin genes, respectively.

ferred gene compared to the endogenous genes (Table 1). In uninduced cells, the transferred gene was expressed at 40–50% the level of a single chromosomal gene. This proportion increased to an average of 85% of the level of a single chromosomal gene after hemin induction.

DISCUSSION

We have used the AAV vector system to introduce a human γ -globin gene into human erythroleukemia cells and achieved high-level regulated globin gene expression. The vector (rAAV/HS2/ γ^* /Neo) that included HS2 from the LCR transferred an intact globin gene in all clones analyzed. Each clone contained one or two copies of the unrearranged proviral genome. The level of γ -globin gene expression was nearly equivalent to that of a native γ -globin gene. Analysis of the 5' end of the mRNA demonstrated that the correct mRNA start site was utilized after AAV transduction. Further characterization of the cell lines demonstrated that wild-type AAV was not present and that after adenovirus superinfection, the AAV transducing vector was not rescued or replicated. Our results established that the AAV vector system is potentially useful for genetic therapy of the human hemoglobin disorders.

A substantial body of experimental data has established the capacity of the LCR or portions thereof to generate copy number-dependent, integration position-independent expression of globin genes in cultured cells and transgenic animals (10, 16, 17). The LCR appears to have at least two functions: (i) to establish an active chromatin structure (43) and (ii) to enhance the level of globin gene expression (11–15, 37). HS2 from the LCR exhibits both of these functions and was therefore used in our experiments. The 400-bp element used to create our vector provides sufficient control to give a level of globin gene expression nearly equivalent to that of a chromosomal globin gene. Most strategies for gene transfer

give concatameric arrays of the integrated globin gene flanked on both sides by LCR elements, whereas the AAV vector integrates one or two copies of the globin gene. Thus, our data establish that a single LCR element adjacent to the globin gene is sufficient for high-level globin gene expression. The role of Neo^R selection in ensuring integration into an active chromatin domain remains to be determined.

Two features of AAV are particularly attractive for human gene therapy applications. The ITRs appear to be devoid of transcriptional regulatory elements (20, 21), reducing the risk for insertional activation of protooncogenes, a recognized mechanism of oncogenesis caused by murine retroviruses. The second feature is the site-specific integration of wild-type AAV into the long arm of chromosome 19 (27, 28). Recent analysis has shown that the junction of several independent integrants lies within a 100-bp region at 19q13.4–ter (28). Site-specific integration of recombinant AAV vectors would further reduce the risk of insertional mutagenesis and could provide a consistent chromosomal environment for expression of the transferred gene. Insertion site analysis of the recombinant AAV genome in our clones suggested random integration. Preliminary studies with similar AAV vectors have also been analyzed for targeted integration. Our results are consistent with these data, which suggest that sequences in addition to the viral terminal repeats may be required for site-specific integration (X.X., X. Zhu, and R.J.S., unpublished observations). However, it is important to note that a major difference between wild-type integration and the AAV vectors characterized in this paper is that we selected for vector integration (Neo^R colonies). What role, if any, this may play (random vs. targeted integration) remains to be determined. Further characterization of AAV integration will ultimately determine whether AAV vectors can be engineered to carry the globin gene and still retain site-specific integration.

The ability of AAV to infect primary hematopoietic stem cells will determine its applicability to human gene therapy. Transduction frequencies of 50–80% have been achieved in tissue culture cells using recombinant vectors lacking the replication function that are free of helper virus (21). Infection of primary mouse hematopoietic progenitors has been reported by using earlier generation vectors that retained the replication function (33). Subsequent work has shown that elimination of this function substantially enhances transduction frequency (21). Our work suggests that recombinant AAV vectors can be used effectively for the transfer of globin genes into human cells. Moreover, an identical AAV vector construct carrying B19 viral sequences was infectious for erythroid progenitor cells from human bone marrow (35). Thus, the available data suggest that the AAV globin vectors will be useful in transferring genes into primary hematopoietic cells.

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Table 1. Expression of AAV transduced γ^* -globin gene relative to endogenous γ -globin gene

Clone	Uninduced, expression	Induced, expression
1	60	100
2	25	40
3	30	60
4	50	100
5	40	110
6	45	80
7	45	60
Polyclonal	40	85

Percentage expression was calculated as the ratio of the signal intensities of the RNA transcripts from the transduced and endogenous γ -globin genes (see Fig. 4) multiplied by twice the ratio of the δ -globin (endogenous) to γ^* -globin (transduced) DNA signals (see Fig. 2). The factor of two corrects for the endogenous γ -globin genes. Densitometric analyses were performed to obtain these ratios.

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